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(54) Title: CARCINOEMBRYONIC ANTIGEN (CEA) LACKING A SIGNAL PEPTIDE, NUCLEIC ACID ENCODING IT AND FUSION OF CEA WITH A T CELL EPITOPE AND THEIR USE FOR THE TREATMENT AND/OR PROPHYLAXIS OF CANCER

(57) Abstract: The present invention relates to a nucleic acid sequence which encodes a carcino embryonic antigen characterized in that said nucleic acid sequence does not encode a signal peptide. The present invention also relates to fusion protein comprising a carcino embryonic antigen and a T cell epitope.

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CARCINOEMBRYONIC ANTIGEN (CEA) LACKING A SIGNAL PEPTIDE, NUCLEIC ACIDS ENCODING IT AND A FUSION OF CEA WITH A T CELL EPITOPE AND THEIR USE FOR THE TREATMENT AND/OR PROPHYLAXIS OF CANCER

The present invention relates to a nucleic acid sequence which encodes a carcino embryonic antigen characterized in that said nucleic acid sequence does not encode a signal peptide. The present invention also relates to fusion protein comprising a carcino embryonic antigen and a T cell epitope.

The invention also relates to nucleic acid sequence which encodes such fusion proteins, to a vector for expressing the different nucleic acid sequence, to a viral particle and a host cell, and to a composition which comprises them. Finally, the invention is also directed towards their therapeutic use and to a method of treatment which implements them. The present invention is particularly useful, within the context of gene therapy, for an application with respect, to proliferative diseases.

Gene therapy is defined as being the transfer of genetic information into a host cell or organism. The first protocol applied to man was initiated in the United States, in September 1990, on a patient who was genetically immunodeficient on account of a mutation which affected the gene encoding Adenine Deaminase (ADA). The relative success of this first experiment encouraged the development of this approach for a variety of diseases, including both genetic diseases (with the aim of correcting the malfunction of a defective gene) and acquired diseases (cancers, infectious diseases, such as AIDS, etc.). This technology has experienced a large number of developments since then, including cancer vaccination, which uses genes whose expression products are able to induce an immune response directed against tumoral cells antigens, thereby leading to the destruction of such cells.

Colon cancer accounts for 10–15% of cancer deaths in the industrialized world, second only to lung cancer. Despite improvements in surgery, chemotherapy and radiotherapy, survival rates are only moderate (40% survive longer than 5 years) and have remained essentially unchanged for the last 20 years. Like many solid tumors, colon cancer may relapse after surgery, probably because of the persistence of micrometastases.

Carcinoembryonic antigen (CEA, also called CEACAM5 3) is a 180 kDa GPI-linked membrane glycoprotein expressed extensively on most human colon, gastric, and

pancreatic carcinomas, as well as on approximately 70% of nonsmall-cell lung carcinomas and 50% of breast cancers. CEA is also expressed on foetal gut tissue and to some extent on normal colonic epithelium.(1,3) It has an approximately 70% homology with nonspecific cross-reacting antigen (NCA), expressed in normal granulocytes.(4) CEA is synthesized as a precursor protein (depicted in SEQ ID NO: 1) with N- and C-terminal signal peptides. These signal peptides target the protein through the endoplasmic reticulum (ER), where it is heavily glycosylated, and subsequently to the cell membrane. Both signal peptides are removed during post-translational processing. The mature protein contains an N-terminal variable region (107 amino acids) and three sets of constant Ig-like repeat regions (178 amino acids each); the C-terminal signal peptide has been replaced by a glycosylphosphatidylinositol (GPI) membrane anchor. The main clinical use of serum CEA has been in postoperative surveillance of colon cancer.

The immunogenicity of endogenous CEA is poor(6,7) Some reports of CEA:anti-CEA complexes in patients exist,(8,9) but others claim that such findings are artefactual. (10,11) Evidence of T-cell responses is scarce, although a response of patient T cells to CEA has been achieved by in vitro immunization with an anti-idiotypic antibody that mimics CEA.(7,12) CEA is expressed extensively on colon carcinomas, and thus constitutes a good potential target for immunotherapy despite its poor immunogenicity. Vaccines using recombinant CEA (rCEA) produced in vaccinia or baculovirus vectors have been used in several studies. Mice immunized with rCEA developed antibody, DTH, lymphoproliferative and cytotoxic responses, and were protected against challenge with murine colon carcinoma cells expressing human CEA.(13) Patients with colon cancer immunized with rCEA mounted a cytotoxic T lymphocyte (CTL) response against tumor cells loaded with CEA peptides.(14) Patients with colon cancer immunized with rCEA mounted both antibody and T-cell responses against CEA that were enhanced by adding granulocyte/macrophage-colony-stimulating factor (GMCSF). (15)

Unlike several infectious diseases, which are well controlled by humoral responses, solid tumors such as colon carcinoma are likely to require cellular responses for effect. Studies in animals reveal that tumor regression is more often associated with cellular than with humoral immunity.(6) While peptide, protein or glycoprotein

vaccines elicit mainly antibody responses, DNA vaccines allow presentation of antigen to the immune system in several ways and have the potential of inducing strong cellular responses. DNA vaccines have been shown to stimulate all three arms of the immune system, that is, antibody, helper T, and cytotoxic T-cell (CTL) responses, (16) to produce immunity in several disease models such as influenza,(17,18) HIV-1,(19,20) and hepatitis B,(21) and to protect animals from subsequent challenge.(18) The mechanisms are considered to involve antigen presentation by both myocytes and antigen-presenting cells (APCs), as well as crosspriming between the two MHC paths. Promising effects have been seen in studies using CEA plasmid DNA vaccines. Humoral, proliferative, and DTH responses were observed in pig-tailed macaques immunized with CEA DNA vaccines,(22) and dogs immunized with CEA DNA vaccines mounted humoral and proliferative responses.(23) Protective effects were also seen in tumor challenge experiments after CEA DNA vaccination in mice . However, there is still a need to improve gene therapy treatment of cancer by CEA DNA vaccination. This problem is solved by the present invention.

The present invention provides new nucleic acid sequences as well as new polypeptides which derive from nucleic acid sequence which encodes CEA. These new sequences and polypeptides induce a more efficient immune response against tumor cells expressing CEA than previously available CEA sequences or polypeptides.

According to a first embodiment, the present invention concerns a nucleic acid sequence which encodes a carcinoembryonic antigen characterized in that said nucleic acid sequence does not encode a signal peptide.

As used herein CEA refers to the Carcinoembryonic antigen, in a preferred embodiment to a protein comprising all or part of an amino acid sequence which is substantially as depicted in SEQ ID NO: 2 and in a more preferred embodiment to an amino acid sequence comprising all or part of an amino acid sequence as depicted in SEQ ID NO: 2. In an even more preferred embodiment, CEA refers to the amino acid sequence depicted in SEQ ID NO: 2.

The term "substantially" refers to a degree of identity with said SEQ ID NO: 2 sequence which is greater than 70%, advantageously greater than 80%, preferably

greater than 90% and, very preferably greater than 95%. Still more preferably, the polypeptide comprises the amino acid sequence depicted in the SEQ ID NO: 2 sequence identifier..

This preferred embodiment can of course be combined with one or more additional mutation(s) at any site in the molecule. Preferably, the additional modification(s) do not significantly affect the antigenicity of the CEA according to the invention. It is pointed out that the antigenicity of the mutants can be tested, in particular using the techniques which are described in the examples which follow.

The term "signal peptide" refers to an amino or carboxy terminal polypeptide preceding the secreted mature protein. The signal peptide is cleaved from and is therefore not present in the mature protein. Signal peptides have the function of directing and translocating secreted proteins across cell membranes. Signal peptide is also referred to as signal protein. For example such signal peptides are depicted in SEQ ID NO: 1 from residue met in position 1 to residue Ala in position 34 and from residue Gly in position 678 to residue Ile in position 702.

The present invention also concerns polypeptide encoded by the nucleic acid sequence according to the invention previously described.

In a second embodiment, the present invention concerns a fusion protein comprising a carcinoembryonic antigen and a T cell epitope.

A "fusion protein" refers to a chimeric molecule formed by the joining of two or more compounds through a bond formed between one moiety and another moiety. For purposes of this invention, one moiety is a polypeptide. The bond between the polypeptide and the other moiety may be covalent or noncovalent. An example of a covalent bond is the chemical coupling of two polypeptides to form peptide bond. Examples of non-covalent bond are hydrogen bonds, electrostatic interactions and van der Waal's forces.

If the bond is a peptide bond the fusion protein may be expressed as a single polypeptide from a nucleic acid sequence encoding a single contiguous fusion protein. According to a preferred embodiment of the invention, the T cell epitope is linked to the N terminal extremity of the carcinoembryonic antigen.

As used herein the term "T-cell epitope" refers to a feature of a peptide structure which is capable of inducing T-cell immunity towards the peptide structure or

an associated hapten. In this regard, it is accepted in the art that T-cell epitopes comprise linear peptide determinants that assume extended conformations within the peptide-binding cleft of MHC molecules, (Unanue et al., Science (1987) 236:551-557). Conversion of polypeptides to MHC class II-associated linear peptide determinants (generally between 5-14 amino acids in length) is termed "antigen processing" which is carried out by antigen presenting cells (APCs). More particularly, a T-cell epitope is defined by local features of a short peptide structure, such as primary amino acid sequence properties involving charge and hydrophobicity, and certain types of secondary structure, such as helicity, that do not depend on the folding of the entire polypeptide. Further, it is believed that short peptides capable of recognition by helper T-cells are generally amphipathic structures comprising a hydrophobic side (for interaction with the MHC molecule) and a hydrophilic side (for interacting with the T-cell receptor), (Margalit et al., Computer Prediction of T-cell Epitopes, New Generation Vaccines Marcel-Dekker, Inc, ed. G. C. Woodrow et al., (1990) pp. 109-116) and further that the amphipathic structures have an .alpha.-helical configuration (see, e.g., Spouge et al., J. Immunol. (1987) 138:204-212; Berkower et al., J. Immunol. (1986) 136:2498-2503).

According to a preferred embodiment, the T cell epitope according to the invention derives from a foreign pathogen. T cell epitopes which derive from foreign pathogens include but are not limited to hepatitis B surface and core antigen helper T cell epitopes), pertussis toxin helper T cell epitopes, tetanus toxoid helper T cell epitopes, measles virus F protein helper T cell epitopes, Chlamydia trachomatis major outer membrane protein helper T cell epitopes, diphtheria toxin helper T cell epitopes, Plasmodium falciparum circumsporozoite helper T cell epitopes, Schistosoma mansoni triose phosphate isomerase helper T cell epitopes, Escherichia coli TraT helper T cell epitopes.

According to a more preferred embodiment, the T cell epitope derives from the tetanus toxoid protein. In an even more preferred embodiment the P2 epitope of the tetanus toxoid protein. In a particularly preferred embodiment the T cell epitope deriving from the P2 epitope of the Tetanus toxoid is substantially as depicted in SEQ ID NO: 3 sequence identifier starting at residue Gln in position 4 and finishing at residue Leu in position 18, and preferably as depicted in SEQ ID NO: 3 sequence

identifier starting at residue Gln in position 4 and finishing at residue Leu in position 18.

A preferred fusion protein according to the invention comprises an amino acid sequence which is substantially as depicted in the SEQ ID NO: 3, and more preferably as depicted in the SEQ ID NO: 3. As mentioned above, it can contain additional mutations.

In a general manner, a polypeptide or a fusion protein according to the invention can be produced either by the conventional methods of chemical synthesis or by recombinant DNA techniques (see, for example, Maniatis et al., 1989, Labora tory Manual, Cold Spring Harbor, Laboratory Press, Cold Spring Harbor, NY). For this reason, the present invention also covers a preparation process in which a nucleic acid sequence encoding said polypeptide or fusion protein is introduced into a cell in order to generate a transformed cell, said transformed cell is cultured under conditions appropriate for enabling said polypeptide or said fusion protein to be produced, and said polypeptide or fusion protein is harvested from the cell culture. The producer cell can be of any origin and, without limitation, a bacterium, a yeast or a mammalian cell, to the extent that the nucleic acid sequence under consideration is either integrated into its genome or integrated into, an appropriate expression vector which is able to replicate. Naturally, the nucleic sequence is placed under the control of transcription and translation signals which enable it to be expressed in the producer cell. Expression vectors and control signals are known to the skilled person. The polypeptide can be recovered from the medium or the cells (after they have been lyzed) and subjected to conventional purification steps (by chromatography, electrophoresis, filtration, immunopurification, etc.).

The present invention also relates to a nucleic acid sequence which encodes a fusion protein according to the invention.

According to the invention, the different nucleic acid sequences according to the invention can be a cDNA or genomic sequence or be of a mixed type. It can, where appropriate, contain one or more introns, with these being of native,

heterologous (for example the intron of the rabbit β-globin gene, etc.) or synthetic origin, in order to increase expression in the host cells. The sequences employed within the context of the present invention can be obtained by the conventional techniques of molecular biology, for example by screening libraries with specific probes, by immunoscreening expression libraries or by PCR using suitable primers, or by chemical synthesis. The mutants can be generated from the native sequences by substituting, deleting and/or adding one or more nucleotides using the techniques of site-directed mutagenesis, of PCR, of digesting with restriction and ligation enzymes, or else by chemical synthesis.

The present invention also relates to a recombinant vector which carries a nucleic acid sequence according to the invention which is placed under. the control of the elements which are required for expressing it in a host cell. The recombinant vector can be of plasmid or viral origin and can, where appropriate, be combined with one or more substances which improve the transfectional efficiency and/or stability of the vector. These substances are widely documented in the literature which is available to the skilled person (see, for example, Feigner et al., 1987, Proc. West. Pharmacol. Soc. 32, 115-121; Hodgson and Solaiman, 1996, Nature Biotechnology 14, 339-342; Remy et al., 1994, Bioconjugate Chemistry,. 5, 647-654). By way of non-limiting illustration, the substances can be polymers, lipids, in particular cationic lipids, liposomes, nuclear proteins or neutral lipids. These substances can be used alone or in combination. A combination which can be envisaged is that of a recombinant plasmid vector which is combined with cationic lipids (DOGS, DC-CHOL, spermine-chol, spermidine-chol, etc.), lysophospholipides (for example Hexadecylphosphocholine) and neutral lipids (DOPE).

According to a preferred embodiment, when cationic lipids are used in the present invention, preference will be given to the cationic lipids describes in EP901463B1 and more preferably pcTG90.

The choice of the plasmids which can be used within the context of the present invention is immense. They can be cloning vectors and/or expression vectors. In a general manner, they are known to the skilled person and, while a number of them are available commercially, it is also possible to construct them or to modify

them using the techniques of genetic manipulation. Examples which may be mentioned are the plasmids which are derived from pBR322 (Gibco BRL), pUC (Gibco BRL), pBluescript (Stratagene), pREP4, pCEP4 (Invitrogene) or p Poly (Lathe et al., 1987, Gene 57, 193-201). Preferably, a plasmid which is used in the context of the present invention contains an origin of replication which ensures that replication is initiated in a producer cell and/or a host cell (for example, the ColE1 origin will be chosen for a plasmid which is intended to be produced in E. coli and the oriP/EBNA1 system will be chosen if it desired that the plasmid should be self-replicating in a mammalian host cell, Lupton and Levine, 1985, Mol. Cell. Biol. 5, 2533-2542; Yates et al., Nature 313, 812-815). The plasmid can additionally comprise a selection gene which enables the transfected cells to be selected or identified (complementation of an auxotrophic mutation, gene encoding resistance to an antibiotic, etc.). Naturally, the plasmid can contain additional elements which improve its maintenance and/or its stability in a given cell (cer sequence, which promotes maintenance of a plasmid in monomeric form (Summers and Sherrat, 1984, Cell 36, 1097-1103, sequences for integration into the cell genome).

With regard to a viral vector, it is possible to envisage a vector which is derived from a poxvirus (vaccinia virus, in particular MVA, canarypoxvirus, etc.), from an adenovirus, from a retrovirus, from a herpesvirus, from an alphavirus, from a foamy virus or from an adenovirus-associated virus.

According to a preferred embodiment, the viral vector according to the invention derives from a Modified Vaccinia Virus Ankara (MVA). Methods to produce such MVA vectors are fully described in European patents EP83286 and EP206920., and in Mayr et al. (1975, Infection 3, 6-14) and Sutter et Moss (1992, Proc. Natl. Acad. Sci. USA 89, 10847-10851). According to a more preferred embodiment, the nucleic acid sequence according to the invention may be inserted in a deletion selected from the group comprising deletion I, II, III, IV, V and VI of the MVA vector and even more preferably in deletion III (Meyer et al., 1991, J. Gen. Virol. 72, 1031-1038 ; Sutter et al., 1994, Vaccine 12, 1032-1040).

Retroviruses have the property of infecting, and in most cases integrating

into, dividing cells and in this regard are particularly appropriate for use in relation to cancer. A recombinant retrovirus according to the invention generally contains the LTR sequences, an encapsidation region and the nucleic acid sequence according to the invention, which is placed under the control of the retroviral LTR or of an internal promoter such as those described below. The recombinant retrovirus can be derived from a retrovirus of any origin (murine, primate, feline, human, etc.) and in particular from the M0MuLV (Moloney murine leukemia virus), MVS (Murine sarcoma virus) or Friend murine retrovirus (Fb29). It is propagated in an encapsidation cell line which is able to supply in trans the viral polypeptides gag, pol and/or env which are required for constituting a viral particle. Such cell lines are described in the literature (PA317, Psi CRIP GP + Am-12 etc.). The retroviral vector according to the invention can contain modifications, in particular in the LTRs (replacement of the promoter region with a eukaryotic promoter) or the encapsidation region (replacement with a heterologous encapsidation region, for example the VL3O type) (see French applications 94 08300 and 97 05203)

Preference will be also given to using an adenoviral vector which lacks all or part of at least one region which is essential for replication and which is selected from the El, E2, E4 and L1-L5 regions in order to avoid the vector being propagated within the host organism or the environment. A deletion of the El region is preferred. However, it can be combined with (an)other modification(s)-/deletion(s) affecting, in particular, all or part of the E2, E4 and/or L1-L5 regions, to the extent that the defective essential functions are complemented in trans by means of a complementing cell line and/or a helper virus. In this respect, it is possible to use second-generation vectors of the state of the art (see, for example, international applications WO-A-94/28l52 and WO-A-97/041l9). By way of illustration, deletion of the major part of the El region and of the E4 transcription unit is very particularly advantageous. For the purpose of increasing the cloning capacities, the adenoviral vector can additionally lack all or part of the nonessential E3 region. According to another alternative, it is possible to make use of a minimal adenoviral vector which retains the sequences which are essential for encapsidation, namely the 5' and 3' ITRs (Inverted Terminal Repeat), and the encapsidation region. The various

adenoviral vectors, and the techniques for preparing them, are known (see, for example, Graham and Prevect, 1991, in Methods in Molecular Biology, Vol 7, p 109-128; Ed: E.J. Murey, The Human Press mc).

Furthermore, the origin of the adenoviral vector according to the invention can vary both from the point of view of the species and from the point of view of the serotype. The vector can be derived from the genome of an adenovirus of human or animal (canine, avian, bovine, murine, ovine, porcine, simian, etc.) origin or from a hybrid which comprises adenoviral genome fragments of at least two different origins. More particular mention may be made of the CAV-I or CAV-2 adenoviruses of canine origin, of the DAV adenovirus of avian origin or of the Bad type 3 adenovirus of bovine origin (Zakharchuk et al., Arch. Virol., 1993, 128: 171-176; Spibey and Cavanagh, J. Gen. Virol. 1989, 70: 165-172; Jouvenne et al., Gene, 1987, 60: 21-28; Mittal et al., J. Gen. Virol., 1995, 76: 93-102). However, preference will be given to an adenoviral vector of human origin which is preferably derived from a serotype C-adenovirus, in particular a type 2 or 5 serotype C adenovirus.

An adenoviral vector according to the present invention can be generated in vitro in Escherichia coli (E. coli) by ligation or homologous recombination (see, for example, international application WO-A-96/17070) or else by recombination in a complementing cell line.

The elements required for expression consist of all the elements which enable the nucleic acid sequence to be transcribed into RNA and the mRNA to be translated into polypeptide. These elements comprise, in particular, a promoter which may be regulable or constitutive. Naturally, the promoter is suited to the chosen vector and the host cell. Examples which may be mentioned are the eukaryotic promoters of the PGK (phosphoglycerate kinase), MT (metallothionein; McIvor et al., 1987, Mol. Cell Biol. 7, 838-848), α-1$_a$antitrypsin, CFTR, surfactant, immunoglobulin, β-actin (Tabin et al., 1982, Mol. Cell Biol. 2, 426-436) and SRa (Takebe et al., 1988, Mol. Cell Biol. 8, 466-472) genes, the early promoter of the SV4O virus (Simian virus), the LTR of RSV (Rous sarcoma virus), the HSV-I TK promoter, the early promoter of

the CMV virus (Cytomegalovirus)., the p7.5K pH5R, pK1L, p28 and pI1 promoters of the vaccinia virus, and the E1A and MLP adenoviral promoters. The promoter can also be a promoter which stimulates expression in a tumor or cancer cell. Particular mention may be made of the promoters of the MUC-I gene, which is overexpressed in breast and prostate cancers (Chen et al., 1995, J.. Clin. Invest. 96, 2775-2782), of the CEA (standing for carcinoma embryonic antigen) gene, which is overexpressed in colon cancers (Schrewe et al., 1990, Mol. Cell. Biol. 10, 2738-2748) of the tyrosinase gene, which is overexpressed in melanomas (Vile et al., 1993, Cancer Res. 53, 3860-3864), of the ERBB-2 gene, which is overexpressed in breast and pancreatic cancers (Harris et al., 1994, Gene Therapy 1, 170-175) and of the α-fetoprotein gene, which is overexpressed in liver cancers (Kanai et al., 1997, Cancer Res. 57, 461-465). The cytomegalovirus (CMV) early promoter and the is very particularly preferred.

When a vector deriving from a Vaccinia Virus (as for example MVA vector) is used, the promoter of the thymidine kinase 7.5K gene is particularly preferred.

The necessary elements can furthermore include additional elements which improve the expression of the nucleic acid sequence according to the invention or its maintenance in the host cell. Intron sequences, secretion signal sequences, nuclear localization sequences, internal sites for the reinitiation of translation of IRES type, transcription termination poly A sequences, tripartite leaders and origins of replication may in particular be mentioned. These elements are known to the skilled person.

The recombinant vector according to the invention can also comprise one or more additional genes of interest, with it being possible for these genes to be placed under the control of the same regulatory elements (polycistronic cassette) or of independent elements. Genes which may in particular be mentioned are the genes encoding interleukins IL-2, IL-4, IL-7, IL-10 and IL-12, interferons, tumor necrosis factor (TNF), colony stimulating factors (CSF), in particular GM-CSF, and factors acting on angiogenesis (for example PAI-1, standing for plasminogen activator inhibitor). In one particular embodiment, the recombinant vector according to the invention comprises the gene of interest encoding IL-2 or encoding interferon γ (INFγ).

The present invention also relates to a viral particle which comprises a recombinant vector according to the invention. Such a viral particle can be generated from a viral vector using any technique which is conventional in the field of the art. The viral particle is propagated in a complementing cell which is suited to the deficiencies of the vector. With regard to an adenoviral vector, use will, for example, be made of the 293 cell line, which was established using human embryonic kidney cells and which efficiently complements the El function (Graham et al., 1977, J. Gen. Virol. 36, 59-72), of the A549-E1 cell line (Imler et al., 1996, Gene Therapy 3, 75-84) or of a cell line which permits double complementation (Yeh et al., 1996, J. Virol. 70, 559-565; Krougliak and Graham, 1995, Human Gene Therapy 6, 1575-1586; Wang et al., 1995 Gene Therapy 2, 775-783; international application WO 97/04119). It is also possible to employ helper viruses to at least partially complement the defective functions. A complementing cell is understood as being a cell which is able to supply in trans the early and/or late factors which are required for encapsidating the viral genome in a viral capsid in order to generate a viral particle which contains the recombinant vector. Said cell may not be able to complement all the defective functions of the vector on its own and, in this case, can be transfected/transduced with a vector/helper virus which supplies the additional functions.

The invention also relates to a process for preparing a viral particle, in which process:

(i) a recombinant vector according to the invention is introduced into a complementing cell which is able to complement said vector in trans, so as to obtain a transfected complementing cell,

(ii) said transfected complementing cell is cultured under conditions which are appropriate for enabling said viral particle to be produced, and

(iii) said viral particle is recovered from the cell culture.

While the viral particle can of course be recovered from the culture supernatant, it can also be recovered from the cells. One of the commonly employed methods consists in lysing the cells by means of consecutive freezing/thawing cycles in order to collect the virions in the lysis supernatant. The virions can then be amplified and purified using the techniques of the art (chromatographic method, method of ultra- centrifugation, in particular through a cesium chloride gradient, etc.).

The present invention also relates to a host cell which comprises a nucleic acid sequence or a recombinant vector according to the invention, or is infected with a viral particle according to the invention. For the purposes of the present invention, a host cell consists of any cell which can be transfected with a recombinant vector or can be infected with a viral particle, as defined above. A mammalian cell, in particular a human cell is very particularly suitable. The cell can comprise said vector in a form which is or is not (episome) integrated into the genome. The cell can be a primary or tumor cell of any origin, in particular an hematopoietic cell (totipotent stem cell, leukocyte, lymphocyte, monocyte or macrophage, etc.), muscle cell (satellite cell, myocyte, myoblast, smooth muscle cell, etc.), cardiac cell, pulmonary cell, tracheal cell, hepatic cell, epithelial cell or fibroblast.

The present invention, also relates to a composition which comprises a polypeptide, a fusion protein, a nucleic acid sequence, a recombinant vector, a viral particle or a host cell according to the invention in combination with a pharmaceutically acceptable excipient

The present invention furthermore relates to a composition which comprises a polypeptide or a fusion protein according to the invention and a polypeptide of interest. Of these polypeptides of interest, particular mention may be made of .interleukins IL-2, IL-4, IL-7, IL-10 and IL-12, interferons, tumor necrosis factor (TNF), colony stimulating factors (CSF), in particular GM-CSF, and factors acting on angiogenesis (for example. PAI-1, standing for plasminogen activator inhibitor).IL-2 or INFγ are very particularly envisaged.

The composition can also be based on nucleic acid sequences which enable the above polypeptides to be expressed within the host cell. The nucleic acid sequences may be carried by one and the same expression vector or by two independent vectors. Said composition can of course comprise viral particles which are generated from (a) viral vector(s) expressing said nucleic acid sequence(s).

The present invention additionally relates to a composition which comprises a nucleic acid sequence according to the invention and a second nucleic acid sequence of interest which encodes a polypeptide selected from IL-2 and INFγ.

A composition according to the invention is more specifically intended for the preventive or curative treatment of diseases by means of gene therapy and is more specifically aimed at proliferative diseases (cancers, tumors).

A composition according to the invention can be made conventionally with a view to administering it locally, parenterally or by the digestive route. In particular, a therapeutically effective quantity of the therapeutic or prophylactic agent is combined with a pharmaceutically acceptable excipient. It is possible to envisage a large number of routes of administration. Examples which may be mentioned are the intragastric, subcutaneous, intracardiac, intramuscular, intravenous, intraperitoneal, intratumor, intranasal, intrapulmonary and intratracheal routes. In the case of these three latter embodiments, it is advantageous for administration to take place by means of an aerosol or by means of instillation. The administration can take place as a single dose or as a dose which is repeated on one or more occasions after a particular time interval. The appropriate route of administration and dosage vary depending on a variety of parameters, for example the individual, the disease to be treated or the gene(s) of interest to be transferred. The preparations based on viral particles according to the invention can be formulated in the form of doses of between 10^4 and 10^{14} pfu (plaque-forming units), advantageously 10^5 and 10^{13} pfu, preferably 10^6 and 10^{12} pfu. As far as the recombinant vector according to the invention is

concerned, it is possible to envisage doses comprising from 0.01 to 100 mg of DNA, preferably from 0.05 to 10 mg, very particularly preferably from 0.5 to 5 mg. A composition based on polypeptides preferably comprises from 0.05 to 10 g, very particularly preferably from 0.05 to 5 g, of said polypeptide. Naturally, the doses can be adjusted by the clinician.

The composition can also include a diluent, an adjuvant or an excipient which is acceptable from the pharmaceutical point of view, as well as solubilizing, stabilizing and preserving agents. In the case of an injectable administration, preference is given to a composition in an aqueous, non-aqueous or isotonic solution. It can be presented as a single dose or as a multidose, in liquid or dry (powder, lyophilizate, etc.) form which can be reconstituted at the time of use using an appropriate diluent.

The present invention also relates to the therapeutic or prophylactic use of a polypeptide, a nucleic acid sequence, a fusion protein, of a recombinant vector, of a viral particle or of a host cell according to the invention for preparing a medicament which is intended for treating the human or animal body by gene therapy or by administering protein which has been produced by the recombinant route. A preferred use consists in treating or preventing cancers, tumors and diseases which result from unwanted cell proliferation. Conceivable applications which may be mentioned are cancers of the breast, of the uterus, of the prostate, of the lung, of the bladder, of the liver, of the colon, of the pancreas, of the stomach, of the esophagus, of the larynx, of the central nervous system and of the blood (lymphomas, leukemia, etc.).

Examples

Methods

CEA DNA constructs

. The plasmid p91023(B) was a kind gift from Nicole Beauchemin and Clifford Stanners, McGill University, Montreal, Canada. It is a 7385 kb plasmid derived from pBR322. It encodes a full-length clone of human CEA (CEA) (accession number

M17303), driven by the adenovirus major late promoter and with an SV40 poly A tail. CEA is 3036 bp long. p91023(B) contains a 5' untranslated region (UTR) (bp 1–96), a 5' signal sequence (ss) (bp 97–198), the CEA region of interest to us, that is, ΔCEA (bp 199–2121), a 30 ss (bp 2121–2205), and a 30 UTR (bp 2206–3036). It contains tetracycline resistance for selection. PCR primers were designed to amplify the CEA gene excluding the signal sequences. To the 5' end of the sense strand primer were added an EcoRI site and a consensus eukaryotic ribosomal binding site. The codon for leu 36 was changed from CTC to CTT to generate a unique HindIII site. To the 5' end of the antisense primer was added a stop codon and a SalI restriction site for subcloning. The PCR product was cloned into the pGEM T-Easy vector (Promega, Madison, WI). The correct product was verified by DNA sequencing (ABI Prism, Perkin-Elmer, Norwalk, CT) and the DCEA insert was recloned into pKCMV using the SalI and EcoRI sites. The pKCMV vector is constructed from pUC8, containing the CMV promoter, but the open reading frame (ORF) of the β-lactamase gene of this plasmid has been replaced by the ORF of the Tn5 aminoglycoside 3'-phosphotransferase (kanamycin resistance gene) in compliance with the Swedish regulatory standards for DNA vaccines administered to humans. The correct product was verified and named pKCMVΔCEA. Paired oligonucleotides encoding the promiscuous helper T-cell epitope QYIKANSKFIGITEL (representing amino acids 830–844 of tetanus toxoid) with HindIII sites at both ends were obtained (Life Technologies), annealed and cloned into the HindIII site of pKCMVΔCEA, thus creating the new sequence MKLQYIKANSKFIGITEL, replacing the original Nterminal signal sequence of CEA. The correct product was verified by DNA sequencing and named pKCEA66.

Immunofluorescence staining and immunoblot analysis of CEA expression in transfected cells

HeLa cells were cultured on coverslips in six-well plates (Nunc, Denmark) in IMDM medium (Life Technologies, Rockville, MD) with 1% l-glutamine, 5% FCS and penicillin/streptomycin (penicillin 50 IU/ml and streptomycin 50 mg/ml, Life Technologies) at 37°C until the cells were 60–80% confluent. For transfections, 5 mg of plasmid DNA was mixed with 5 ml 20% glucose and 2.4 ml polyethyleneimine

(Sigma-Aldrich, St Louis, MO) in 20 ml water and added to 1ml of growth medium before adding to the cells. After transfection, the cells were incubated overnight and the medium was changed. At 36– 40 hours post-transfection, the cells on coverslips were washed three times in PBS and then blow-dried before they were fixed on the cover glass with -20°C acetone–methanol (80:20). The cells were immunostained by a standard double antibody technique using a monkey anti-CEA IgG diluted 1/2000 as primary antibody and a fluorescein-conjugated, affinity purified goat anti-human IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) as secondary antibody, diluted 1/50 in PBS with 0.01% Tween-20 and 10% NGS. Finally, the cells were counterstained for 5 minutes with a 1/2000 dilution of Hoechst 33258 (500 mg/ml in water). For testing expression of the plasmid DNAs in immunoblots, D17 canine osteosarcoma or human 293 cells were transfected in 60mm Petri dishes with 9 mg of p91023(B) (wtCEA), the ΔCEA clones or the pKCMV vector control plasmid using 30 ml Lipofectin (Life- Technologies) in DMEM without serum or antibiotics. After 7 hours later, the medium was exchanged for DMEM with 10 % FBS. At 2 days after transfection, the cells were lysed in SDS and analyzed on immunoblots using standard methods, using the monkey-anti-CEA IgG as primary antibody and a horseradish peroxidaseconjugated goat anti-human IgG for detection using a luminol reagent (Amersham-Pharmacia Biotech, UK).

Quantitation of CEA expression in LS174T tumor cells using an enzyme-linked immunosorbent assay (ELISA)

The growth medium from a 3-day culture of LS174T cells was centrifuged at 12,000 rpm for 15 minutes to remove cell debris and the supernatant was stored at -20°C until use. The cells were counted, washed three times in PBS, and lysed for 30 minutes on ice in RIPA buffer. Growth medium, cell lysate and rCEA (baculovirus expressed recombinant human CEA protein, Protein Science, Meriden, CT) were then serially diluted with PBS containing 0.5% BSA, 2% normal goat serum (NGS) and 0.05% Tween-20, and preincubated for 1 hour at 37°C with monkey-anti-CEA antibodies (SIIDC, Stockholm) diluted at a final concentration of 1/16,000. A 96- well Nunc ImmunoMaxisorb plate was coated with 0.1 mg/well of rCEA antigen diluted in 0.05M Na_2CO_3 pH 9.6 overnight at room temperature. The wells were washed four

times with 0.9% NaCl, 0.05% Tween-20, blocked 2 hours at 37°C with 2% BSA in PBS and then washed four times. The monkey-anti-CEA antibodies, preincubated with appropriate dilutions of growth medium, cell lysate and rCEA, as described above, were added to different wells and incubated for 2 hours at 37°C. The wells were washed four times before the addition of goat-anti-human IgG diluted 1/3000 in PBS with 0.5% BSA, 2% NGS and 0.05% Tween-20. The plate was incubated for 2 hours at 37°C. After washing the wells four times, the plate was incubated for 15 minutes at 37°C with phosphatase substrate (Sigma Diagnostics Inc.) diluted according to the manufacturer's instructions. The reaction was stopped by adding 1M NaOH and the plate was read at 405 and 650nm in a double-beam ELISA reader. Unspecific binding of monkey-anti-CEA to the plate and goat-antihuman IgG to the antigen were tested as negative controls. Noninhibited monkey-anti-CEA diluted 1/16,000 was used as a positive control. CEA expression in cell lysates as well as in the supernatant medium was normalized to the number of cells in the original culture. CEA expression on the tumor cells and in the medium was calculated using a standard inhibition curve created by incubating known amounts of rCEA with monkey anti-CEA IgG.

Immunization of C57BL/6 mice with CEA plasmids

Three groups of ten 10–12-week-old C57BL/6 (H2b) mice were subjected to immunization with plasmid DNA as described below. Plasmid DNAs were transformed into E. coli DH5α (Life Technologies), and purified using Qiagen endotoxin-free Maxi or Mega plasmid preparation kits (Qiagen, Germany) and dissolved at 2 mg/ml in water. One group was immunized intramuscularly (i.m.) in the quadriceps muscle with 100 mg of plasmid pKCEA66, one with 100 mg i.m. of plasmid p91023(B) and 10 with 100 mg i.m. of plasmid pKCMV. After 28 days the mice received a second dose of 100 mg of the respective DNAs, administered in the same way. On day 69 the mice in each group received gene gun immunization (see below) with a total of 5 mg of the respective DNAs administered epidermally at two different locations on the abdomen. Blood samples were taken on days 45 and 88 and before the mice were killed on day 103. Sera were used for tests of humoral immune responses by ELISA while spleen

cells were used for testing T-cell and cytokine responses and for transplantation to SCID mice prior to tumor challenge.

Preparation of DNA-loaded gold beads for gene-gun immunization

Aliquots of 20.7, 20.4 and 21.7 mg gold beads (Microcarrier 1.0 mm, Bio-Rad Laboratories) were placed in three 1.5 ml microcentrifuge tubes. The pKCEA66, p91023(B) and pKCMV plasmids were added at a loading rate of 5.07, 5.15 and 4.84 mg DNA/mg gold, respectively. Each aliquot, containing approximately 2.5 mg DNA, was prepared for injection by standard techniques and was administered using a helium pulsed Accell device (Geniva Inc., Middletown, WI).

ELISA measurement of isotype-specific antibody responses after immunization of C57BL/6 mice

Coating of 96-well Nunc ImmunoMaxisorb plates was performed overnight at room temperature with with 0.1 mg/well of rCEA antigen (rCEA2) diluted in 0.05M Na_2CO_3 pH 9.6. The wells were washed four times with 0.9% NaCl, 0.05% Tween-20 and then blocked for 2 hours at 37°C with 2% bovine serum albumin (BSA) in PBS. After four washes, serum samples were applied, diluted 1/50 and 1/250 in Buffer A (0.5% BSA and 0.05% Tween-20 in PBS), and incubated for 2 hours at 37°C followed by four washes. Goat anti-mouse IgG1, IgG2a, IgG2b, IgG3 or IgA (Caltag) diluted 1/1000 in Buffer A was added and the plates were incubated 90 minutes at 37°C. After washing the wells four times the plates were incubated 90 minutes at 37°C with alkaline phosphatase conjugated Affinipure rabbit-anti-goat Ig (DAKO) diluted 1/2000 in Buffer A. The wells were washed four times before OPD substrate (o-phenylenediamine dihydrochloride, Sigma, St Louis, MO) diluted in citratephosphate buffer pH 5.0 and H_2O_2 (one tablet in 10 ml buffer and 3 ml H_2O_2) was added and the plates were incubated 20 minutes at room temperature. The reaction was stopped with 2.5M H_2SO_4 and read at 490 and 650nm in a double-beam ELISA reader. Unspecific binding of the anti-mouse antibodies to the antigen was tested as a control. Monkey-anti-CEA IgG and a known positive serum were used as positive controls. To reveal monkey-anti-CEA binding, a 1/20,000 dilution of an affinity-purified goat-anti-human Ig horseradish peroxidase conjugate (BioRad) was used.

Dose titration of human CEA-expressing LS174T cells for tumor challenge experiments

The CEA secreting human colorectal cancer cell line LS174T was grown at 37°C in Parker 199 medium (SBL Vaccin AB, Stockholm, Sweden) with l-glutamine (Life Technologies Ltd), 10% fetal calf serum (FCS) and penicillin/streptomycin (Life Technologies Ltd). To determine the cell dose for subsequent tumor cell challenges a titration with different concentrations of LS174T tumor cells was performed in 10 SCID mice (scid/scid C.B.-17). The mice were divided in two groups and inoculated subcutaneously with three doses of tumor cells each. Five mice received 10^3, 10^4 and 10^5 cells at different sites and five mice received 10^5, 10^6 and 3 10^6 cells. Based on this titration, each SCID mouse received challenge doses of 10^6, 5 10^5 and 10^5 LS174T cells subcutaneously at three separate locations 1 day after receiving an intraperitoneal graft of B-, T-, or B+T-cell-depleted spleen cells from C57BL/6 mice previously immunized with DNA constructs expressing either wtCEA (p91023(B)), tetΔCEA (pKCEA66) or control plasmid (pKCMV).

Negative selection of immune C57BL/6 spleen cells for SCID transplantation

The C57BL/6 mice were killed on day 103 and blood samples and spleens were collected. Spleens were mashed in 5–10 ml medium with 10% FCS. Cell suspensions were centrifuged briefly to remove large cell aggregates, and the supernatants were centrifuged a second time. Cells were resuspended in 5ml medium with 10% FCS, counted, and diluted to 2 10^6 cells per ml. T-cell proliferation tests were made on a small portion of the cells. Spleen cells from each group of mice immunized with the same plasmid were pooled and divided into three equal aliquots for negative selection of effector cells using Dynabeads (Dynal AS, Oslo, Norway). In the first aliquot, B cells were removed using B220 antibody-coated beads, in the second aliquot CD4+ and CD8+ cells (TH and TC cells) were removed using L3T4and Lyt2 antibody-coated beads and in the third aliquot both B cells, CD4+ and CD8+ cells were removed using a combination of all three beads. The antibodies for coating were rat antimouse monoclonal antibodies. The cell separation was performed according to the manufacturer's protocols. Controls of cell depletion efficacy by FACS revealed that

92–97% of the targeted cells had been removed. Following removal of the magnetic beads, the cell suspension was concentrated to a proper volume and injected intraperitoneally (i.p.) in SCID mice. Within each group of 10 SCID mice, three mice received spleen cells depleted for B cells, three mice received spleen cells depleted for T cells and four mice received T- and B-cell-depleted cells. Within the group receiving spleen cells from pKCEA66- immunized C57BL/6, the spleen cell numbers were as follows: B-cell depleted, $30.8.10^6$ cells per mouse; T-cell depleted, $64.8\ 10^6$ cells per mouse; B- and T-cell depleted, $19.1\ 10^6$ cells per mouse. Within the group receiving spleen cells from p91023(B) (wtCEA)-immunized C57BL/6, the spleen cell numbers were as follows: B-cell depleted, $24.4\ 10^6$ cells per mouse; T-cell depleted, $44.3\ 10^6$ cells per mouse; B- and T-cell depleted, $14.4\ 10^6$ cells per mouse. Within the group receiving spleen cells from pKCMV (vector)-immunized C57BL/6, the spleen cell numbers were as follows: B-cell depleted, $55.6\ 10^6$ cells per mouse; T-cell depleted, $53.6\ 10^6$ cells per mouse; B- and T-cell depleted, $15.8\ 10^6$ cells per mouse. A fourth group of SCID mice received no spleen cell transplants. The day after the spleen cells had been injected, the SCID mice were inoculated with LS174T tumor cells subcutaneously in three doses: 10^6, $5\ 10^5$ and 10^5 cells at different sites and the tumor size was measured on days 4, 7, 12 and 15 after tumor challenge. SCID mice were killed on day 15 when the maximum permissible tumor size was reached; however, SCID mice in the group receiving spleen cells from C57BL/6 mice immunized with pKCEA66 were monitored for tumor growth up to 4 weeks.

T-cell stimulation assays

All media used in immunoassays consisted of RPMI 1640 containing 5 or 10% heat-inactivated FCS, 1% penicillin/ streptomycin (final concentration 50 IU/ml and 50 mg/ml, respectively) (Life Technologies), 0.2mM 2-mercaptoethanol, and 10mM sodium pyruvate, unless otherwise noted. Spleen cells from the immunized C57BL/6 mice were cultured in RPMI medium with 10% FCS. The following components were added to 96-well plates in 100 ml medium with 10% FCS: medium alone, ConA (final concentration 5 mg/ml), native CEA (10 mg/ml) and rCEA (10 mg/ml), or recombinant control protein (rCP, 10 mg/ml). Subsequently, $2\ 10^5$ cells per well in another 100 ml medium with 10% FCS was added. Each sample was made in triplicate. Cells were

cultured at 37°C for 5 days. ^{3}H-labelled thymidine (50 ml per well, 1 mCi) was added and the cells were incubated for another 16 hours before they were harvested in a Harvester 96 (Tomtec, CT) and the incorporation of thymidine by the cells was measured in a liquid b-scintillation counter (Wallac Micro Beta TriluxeTM, Turku, Finland). The SI was calculated by dividing the mean cpm values from the triplicate CEA stimulated wells with the mean cpm of the medium control wells.

Results

Wild-type and mutated CEA show different intracellular expression patterns

The DNAs tested for expression in D17 or 293 cells were wtCEA represented by p91023(B), ΔCEA with deleted signal sequences (pKCMVΔCEA) and pKCEA66, where the promiscuous helper T cell epitope QYIKANSKFIGITEL (representing amino acids 830–844 of tetanus toxoid) was inserted at the N terminus of the CEA in pKCMVΔCEA and one clone of a negative vector control (pKCMV). The wtCEA protein, expressed from the p91023(B) plasmid, shows a characteristic expression pattern of a plasma membrane protein, i.e. presence in the ruffled edge and cytoplasmic projections of the cell. In contrast, the majority of the ΔCEA protein was found in aggregates in a perinuclear or cytoplasmic distribution, while only a minority of the ΔCEA protein was observed on the membrane. CEA protein expressed from the pKCEA66 vector gave rise to a homogeneous cytoplasmic fluorescence with marked vacuolization. The pKCMV vector was negative. Cell lysates from transfected cells were analyzed on immunoblots . rCEA protein was used as a positive control and migrated at 130–200 kDa; ΔCEA migrated at 130 kDa, while wtCEA (p91023(B)) had bands both at 130 and 200 kDa. rCEA purified from a baculovirus expression system is differently glycosylated and has a size of 120 kDa with considerable size variation on immunoblots. Eliminating the signal sequences also eliminates glycosylation (since the protein is not translocated to the ER). p91023(B) encodes full-length CEA, which is glycosylated, although overexpression and aberrant intracellular transport in the transfected cells may limit glycosylation of some full-length CEA molecules, explaining the appearance of two bands.

Humoral immune responses in C57BL/6 mice immunized with the pKCEA66 plasmid favor IgG1 over IgG2a.

Humoral immune responses after plasmid DNA immunizations were tested by ELISA for CEA reactive antibodies of isotypes IgG1, IgG2a, IgG2b, IgG3, IgM and IgA. IgG2b-specific responses were similar to IgG2a. The IgG3, IgM and IgA responses were low and did not differ significantly between the groups of immunized mice. The response profiles were carried out with sera collected at the time of killing, on day 103. The antibodies were directed against both native and rCEA. All mice immunized with plasmid pKCEA66 had a strong IgG1 response. The IgG1 response was stronger than any of the other subclasses. Three of 10 mice immunized with plasmid p91023(B) also developed strong IgG1 responses, while none of the mice immunized with the empty plasmid pKCMV had a significant humoral response. The immunization schedule was as described in Methods. These results show that CEA specific humoral immune responses were raised against the wtCEA plasmid p91023(B) as well as against the pKCEA66 plasmid.

C57BL/6 mice immunized with plasmids encoding wtCEA or tetΔCEA (tetanus toxoid P2 epitope/CEA fusion protein encoded pKCEA66) by mount T-cell-specific responses to CEA

Proliferation of lymphocytes in response to CEA antigen following plasmid immunization was analyzed in parallel with the humoral response. A small portion of pooled spleen cells collected from each group of immunized C57BL/6 mice was used for T-cell proliferation assays. Mice immunized with plasmid pKCEA66 developed a cell-mediated response with a mean SI of 6.2. Spleen cells from mice immunized with plasmid p91023(B) had a mean SI of 4.0 and spleen cells from mice immunized with plasmid pKCMV showed no significant reactivity to CEA (SI of 1.2). A SI above 2 was considered positive. Spleen cells from the group immunized with pKCEA66 showed higher activity.

Spleen cells from pKCEA66-immunized C57BL/6 mice reduce both tumor frequency and tumor growth rate in SCID mice after challenge with CEA tumor cells

Spleen cells from the plasmid-immunized C57BL/6 mice were transplanted to SCID mice intraperitoneally, after a negative selection had been performed where B, T or both B and T cells were removed. The SCID mice were then inoculated with human tumor cells in three doses and the tumor growth was measured. The control SCID animals received tumor cells only. The LS174T tumor cells expressed 0.22 pg CEA/cell and secreted 0.41 pg CEA/cell into the medium during 3 days. This indicates a continuous and high expression and secretion of CEA antigen by the cells. The tumor take frequency as well as tumor growth differed significantly ($P<0.01$) between animals receiving spleen cells from pKCEA66-immunized C57BL/6 mice, and animals in all other groups. In the group receiving pKCEA66-immunized spleen cells, only four of 10 animals developed tumors at all, and only in the highest tumor cell dose (10^6 tumor cells). The growth rate and final size of these tumors in the individuals receiving spleen cells from pKCEA66-immunized mice was also strongly diminished. In groups of SCID mice receiving spleen cells from C57BL/6 mice immunized with p91023(B) or the pKCMV vector control, all the tumor take rates were similar and did not differ significantly from the tumors of SCID mice receiving no spleen cells.

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Claims

1. A nucleic acid sequence which encodes a carcinoembryonic antigen characterized in that said nucleic acid sequence does not encode a signal peptide.

2. A nucleic acid sequence according to claim 1, wherein said carcinoembryonic antigen comprises an amino acid sequence substantially as depicted in SEQ ID NO: 2

3. A nucleic acid sequence according to claim 1, wherein said carcinoembryonic antigen comprises an amino acid sequence as depicted in SEQ ID NO: 2.

4. A polypeptide encoded by the nucleic acid sequence according to anyone of claims 1 to 3.

5. A fusion protein comprising:
   - a polypeptide according to claim 4 and
   - a T cell epitope.

6. A fusion protein according to claim 5, wherein the T cell epitope is linked to the N terminal extremity of said polypeptide.

7. A fusion protein according to claim 5, wherein the T cell epitope derives from the tetanus toxoid protein.

8. A fusion protein according to claim 7, wherein the T cell epitope derives from the P2 epitope of the tetanus toxoid protein.

9. A fusion protein according to claim 8, wherein the T cell epitope comprises a amino acid sequence substantially as depicted in SEQ ID NO: 3 sequence identifier starting at residue Gln in position 4 and finishing at residue Leu in position 18.

10. A fusion protein according to claim 9, wherein the T cell epitope comprises an

amino acid sequence as depicted in SEQ ID NO: 3 sequence identifier starting at residue Gln in position 4 and finishing at residue Leu in position 18.

11. A nucleic acid sequence which encodes a fusion protein according to one of claims 5 to 10.

12. A recombinant vector which carries a nucleic acid sequence according to claim 11 or to one of claims 1 to 3 placed under the control of the elements which are required for expressing it in a host cell.

13. A recombinant vector according to claim 12, characterized in that said vector is selected from the group consisting of the plasmid and viral vectors, where appropriate combined with one or more substances which improve(s) the transfectional efficacy and/or the stability of the vector.

14. A recombinant vector according to claim 13, wherein said substance which improve the transfectional efficacy and/or the stability of the vector is selected from the group consisting in cationic lipids, cationic polymers, lysophospholipides and polypeptides.

15. A recombinant vector according to Claim 13, characterized in that said vector is a viral vector which is derived from a pox virus, from an adenovirus, from a retrovirus, from a herpes virus, from an alphavirus, from a foamyvirus or from an adenovirusassociated virus.

16. A recombinant vector according to claim 15, characterized in that said vector derived from a Modified Vaccinia Ankara (MVA) virus.

17. A recombinant vector according to claim 16, characterized in that the nucleic acid sequence according to claim 11 or to anyone of claims 1 to 3 is inserted at a site of a naturally occurring deletion within the MVA genome selected from the group consisting in deletion I, II, III, IV, V and VI.

18. A recombinant vector according to claim 17, wherein the site of the naturally occurring deletion is deletion III.

19. A recombinant vector according to one of claims 12 to 18, characterized in that the elements which are required for the expression comprising a promoter.

20. A recombinant vector according to claims 17 or 18, characterized in that the promoter is the promoter of the thymidine kinase 7.5K gene.

21. A recombinant vector according to claim 15, characterized in that said vector is an adenoviral vector which lacks all or part of at least one region which is essential for replication and which is selected from the EI, E2, E4 and L1-L5 regions.

22. A recombinant vector according to Claim 21, characterized in that said vector is an adenoviral vector which additionally lacks all or part of the non-essential E3 region.

23. A recombinant vector according to claim 12, characterized in that the elements which are required for the expression comprise a promoter, in particular the cytomegalovirus (CMV) early promoter.

24. A recombinant vector according to one of claims 12 to 24, characterized in that it additionally comprises one or more genes of interest which is/are selected from the genes encoding interleukins IL-2, IL-4, IL-7, IL-10 and IL-I2, interferons, tumor necrosis factor (TNF), colony stimulating factors (CSF) and factors acting on angiogenesis.

25. A recombinant vector according to claim 24, characterized in that the gene of interest encodes a polypeptide which is selected from IL-2 and INFy.

26. A process for preparing a viral particle, in which:
a recombinant vector according to one of claims 15 to 25 is introduced into a complementing cell which is able to complement said vector in trans so as to obtain a tranfected complementing cell;
said transfected complementing cell is cultured under conditions which are appropriate for enabling said viral particle to be produced, and
said viral particle is recovered from the cell culture.

27. A viral particle which comprises a recombinant vector according to one of

claims 15 to 25 or was obtained in accordance with the process according to claim 26.

28. A host cell which comprises a nucleic acid sequence according to one of claims 1 to 3, or a nucleic acid sequence according to claim 11, or a recombinant vector according to one of claims 12 to 25, or which is infected with a viral particle according to claim 27.

29. A composition which comprises a polypeptide according to claim 4, a fusion protein according to one of claims 5 to 10, a nucleic acid sequence according to one of claims 1 to 3, a nucleic acid sequence according to claim 11, a recombinant vector according to one of claims 12 to 25, a viral particle according to claim 27, or a host cell according to claim 28, in combination with a pharmaceutically acceptable excipient.

30. Therapeutic or prophylactic use of a polypeptide according to claim 4, a fusion protein according to one of claims 5 to 10, a nucleic acid sequence according to one of claims 1 to 3, a nucleic acid sequence according to claim 11, a recombinant vector according to one of claims 12 to 25, a viral particle according to claim 27, or a host cell according to claim 28 for preparing a medicament which is intended for treating the human or animal body by gene therapy or by administering protein which has been produced by the recombinant route.

31. Use according to claim 30, for preparing a medicament which is intended for treating cancers, tumors and diseases which result from unwanted cell proliferation.

32. Process for preparing a polypeptide according to claim 4, a fusion protein according to one of claims 5 to 10, in which:
a nucleic acid sequence according to one of claims 1 to 3, or a nucleic acid sequence according to claim 11 is introduced into a cell in order to generate a transformed cell
said transformed cell is cultured under conditions which are appropriate for enabling said polypeptide to be produced, and
said polypeptide or said fusion protein is harvested from the cell culture.

SEQUENCE LISTING

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<151> 2003-04-15

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Ile Gln Asn Asp Thr Gly Phe Tyr Thr Leu His Val Ile Lys Ser Asp
            100                 105                 110

Leu Val Asn Glu Glu Ala Thr Gly Gln Phe Arg Val Tyr Pro Glu Leu
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A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C07K14/705 C12N15/12 C12N15/85 C12N15/86 C12N15/62
C12N5/10 A61K38/17 A61K48/00 A61P35/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K. C12N A61K A61P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, EMBASE, SCISEARCH, WPI Data, PAJ

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95/32286 A (MICROGENESYS INC) 30 November 1995 (1995-11-30) pages 9,10 pages 19,20 page 15 examples 4,7	1-4,12, 13,19, 26-32
X	WO 02/40059 A (LOUKINOV DMITRI I ; MINCHEFF MILCHO S (US); ZOUBAK SEGUEI (US); AMERIC) 23 May 2002 (2002-05-23) page 35 example 7	1-4,19, 23,29-31
Y	page 41 - page 56	15-32

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
19 August 2004	06/09/2004

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Domingues, H

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 672 513 A (TERSKIKH ALEXEY ET AL) 30 September 1997 (1997-09-30) column 6 columns 8,9	1-4,12, 13,19, 23,28,32
Y	SLINGLUFF C L JR ET AL: "Phase I trial of a melanoma vaccine with gp100(280-288) peptide and tetanus helper peptide in adjuvant: immunologic and clinical outcomes." CLINICAL CANCER RESEARCH : AN OFFICIAL JOURNAL OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH. OCT 2001, vol. 7, no. 10, October 2001 (2001-10), pages 3012-3024, XP002293206 ISSN: 1078-0432 pages 3102-3 page 3019 page 3020 page 3022 table 5	5-32
Y	GAL LE F-A ET AL: "Lipopeptide-based melanoma cancer vaccine induced a strong MART-27-35-cytotoxic T lymphocyte response in a preclinal study" INTERNATIONAL JOURNAL OF CANCER, NEW YORK, NY, US, vol. 98, no. 2, 2002, pages 221-227, XP002225276 ISSN: 0020-7136 the whole document	5-32
Y	US 4 603 112 A (PAOLETTI ENZO ET AL) 29 July 1986 (1986-07-29) the whole document	15-32
Y	MOINGEON P: "Cancer vaccines" VACCINE, BUTTERWORTH SCIENTIFIC. GUILDFORD, GB, vol. 19, no. 11-12, 8 December 2001 (2001-12-08), pages 1305-1326, XP004313943 ISSN: 0264-410X the whole document	1-32
Y	EP 0 346 710 A (MOLECULAR DIAGNOSTICS INC) 20 December 1989 (1989-12-20) the whole document	1-32

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